APPLICATION NOTE

Response Profiles of Known Autophagy-Modulators Across Multiple Cell Lines: Using Cyto-ID® Autophagy Dye to assess Compound Activity and Toxicity

Kim Luu[1], Wini Luty[2]
[1]IntelliCyt Corporation, Albuquerque, New Mexico, USA • [2]Enzo Life Sciences, Farmingdale, New York, USA

ENZO Cyto-ID® Autophagy Detection Kit (ENZ-51031)
IntelliCyt MultiCyt FL3 Cell Membrane Integrity Kit (90346)

Autophagy has rapidly become an important target in drug discovery. However, current methods to assay autophagy-related responses are not easily adapted to high throughput screening campaigns. This application note demonstrates the use of Enzo's Cyto-ID® Autophagy Dye and IntelliCyt's MultiCyt Cell Membrane Integrity Dye and iQue® Screener as a platform to detect changes to basal autophagy in cell-based assays. The autophagy response is profiled in a screening compatible workflow for 14 known effectors of autophagy across 6 different cell lines in a mix-and-read, no wash assay.

Introduction

Autophagy (in particular, macroautophagy) is a pathway that delivers a portion of a cell's cytoplasm to the lysosome for degradation. Turnover of proteins and long-lived organelles by autophagy are a normal part of the homeostatic activities of a cell. During periods of metabolic stress, such as nutrient limitation, autophagy is upregulated to generate the necessary macromolecules for new synthesis. In addition to its importance in the stress response, autophagy is known to play a role in neurodegenerative, liver, muscle, and cardiac diseases, as well as cancer, aging, and clearance of infections (1). The involvement of autophagy in a wide variety of disorders has made it an attractive target in drug discovery.

The current understanding of the molecular mechanisms of autophagy are well reviewed in the literature (2). In brief, autophagy initiates with the formation of a phagophore, which is a membrane that begins to surround a portion of the cytoplasm (Figure 1). The phagophore develops into a fully enclosed vesicle called an autophagosome that completely sequesters a portion of the cytoplasm. The autophagosome then docks and fuses with a lysosome, creating an autolysosome, and the contents of the vesicle and the vesicle itself are degraded.

Most methods of autophagy detection use quantification or visualization of autophagy-related intracellular markers. A commonly used marker is LC3-II because it is known to uniquely associate with the fully formed autophagosome (3). Microscopy or western blotting can be used to quantify LC3-II as a measure of autophagosome accumulation or depletion in response to a treatment. Engineered cell lines are often used, in which case an LC3/fluorescent protein fusion is overexpressed within the cell. These methods require complex assay development, can be cumbersome in high throughput screenings, and in the case of engineered cell lines, raise questions of physiological disease relevance.

In this application note, the ability of the iQue Screener System to detect changes to basal autophagy is characterized in multiple cell lines. Cyto-ID® Autophagy Dye and IntelliCyt's MultiCyt Cell Membrane Integrity reagent are used to quantify autophagy and cell viability from the same samples. Fourteen known modulators of autophagy, selected because they span multiple mechanisms of action, are shown to cause changes in autophagy dye staining that varies by cell type. Notably some compounds cause significant cell toxicity in addition to perturbations in autophagy. The membrane integrity endpoint measurement is utilized as a primary quality control "flag" whereby samples with less than 80% viability are not evaluated for autophagy as the biological relevance at that point is ambiguous.

The assay platform offers unique advantages for high throughput screening: it is homogeneous, requiring only addition of the dye to your assay and 30 minute incubation. Importantly, this assay allows screening your cellular model of choice without having to engineer a specific cell line. The ability to multiplex and simultaneously detect cell viability in addition to autophagy provides a powerful mechanism for uncovering compound toxicity that could contribute to artifacts in a potential autophagy screen.

Assay Principles

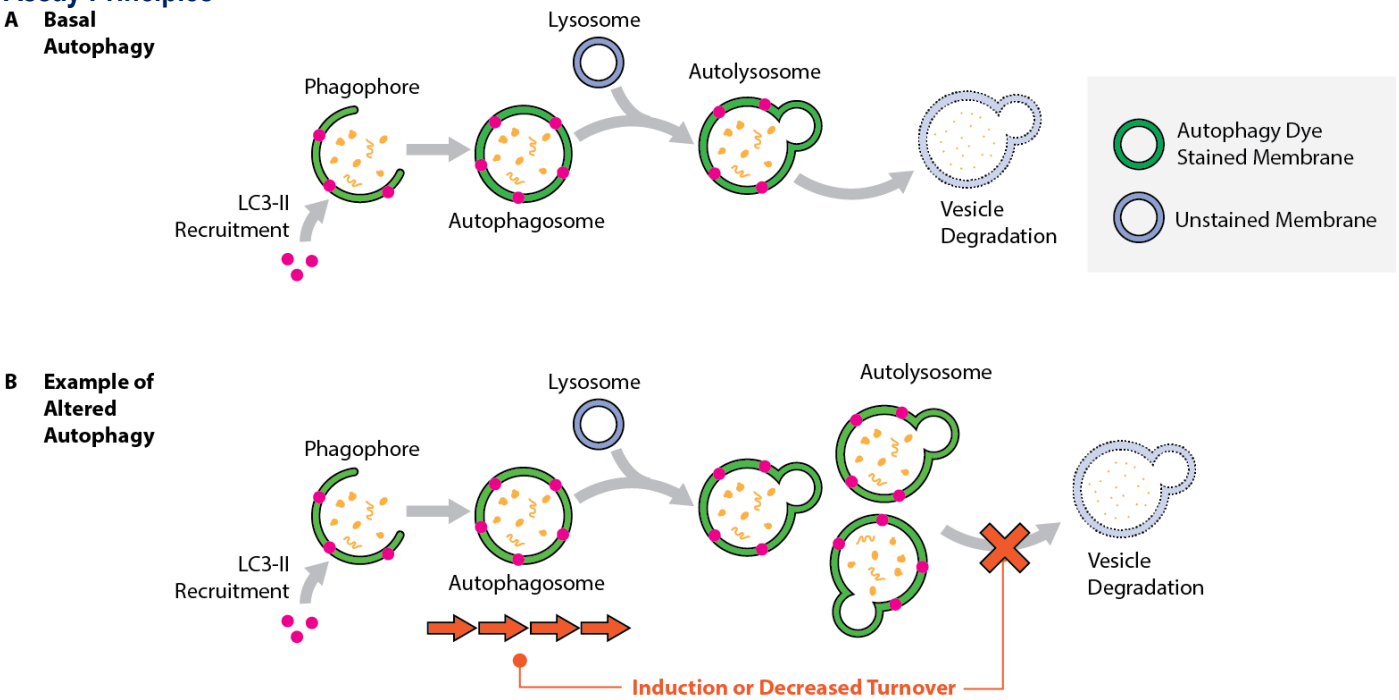

Figure 1. How Cyto-ID® Autophagy Dye works. Cyto-ID® Autophagy Dye stains the membranes of phagophores, autophagosomes, and autolysosomes, with minimal staining of lysosomes. (A) Mammalian cells undergo a basal level of autophagy as a normal homeostatic process and will stain positively with Cyto-ID® Autophagy Dye. (B) Any perturbation that causes a change to the total amount of autophagic compartments will cause cells to stain with more or less Cyto-ID® Autophagy Dye than untreated cells. In the example above, the accumulation of autolysosomes increases the total number of autophagic compartments in the cell and thus causes an increase in autophagy staining. Importantly, the accumulation or depletion of autophagic compartments can be caused by either upregulation or inhibition of steps in the autophagy pathway. Because of this, the Cyto-ID® Autophagy Dye is a gross measure of autophagy perturbation rather than a specific reporter of the magnitude of change to autophagic-flux.

The mechanism of action, measurement and analytical approach for the use of Cyto-ID® Autophagy Dye are explained in Figures 1 and 2. The assay workflow is detailed in Figure 3.

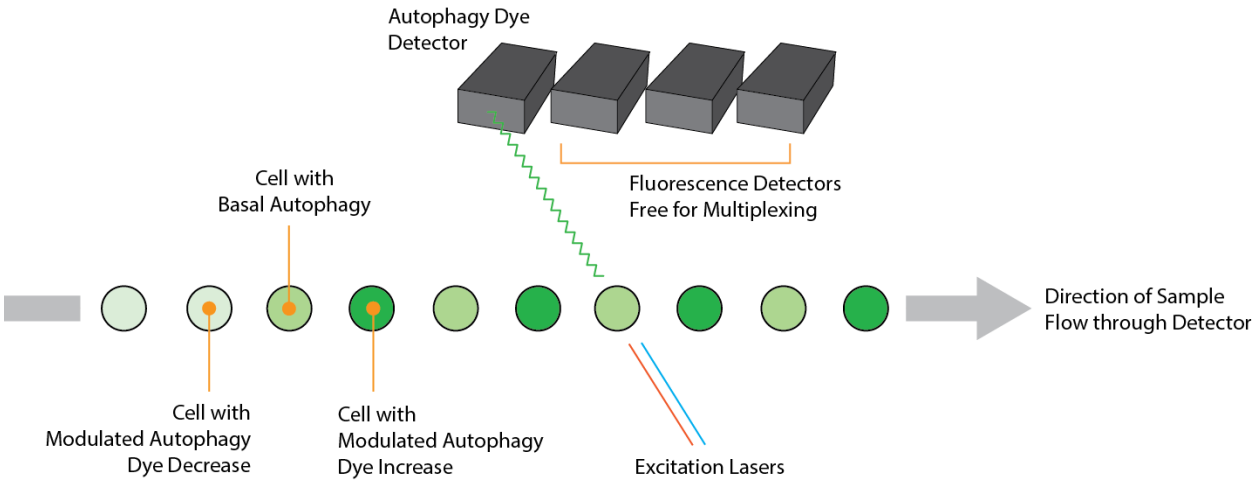

Figure 2. Detection of cells stained with Cyto-ID® Autophagy Dye. After staining samples with Cyto-ID® Autophagy Dye, the iQue screener delivers the samples to a fluorescence detector. The cells within each sample are aligned single-file and per cell fluorescence measurements are made to quantify the amount of autophagy dye retained by the cell. The iQue screener supports detection of 4 simultaneous fluorescence measurements, meaning the autophagy dye can be multiplexed with up to 3 different fluorescent probes, allowing multiplexing of the autophagy readout with other important biological endpoints such as cell membrane integrity.

Once samples are stained with a cocktail containing Cyto-ID® Autophagy Dye and the MultiCyt Cell Membrane Integrity reagent, they are directly read from microtiter plates using the iQue Screener. Samples are drawn from each well and fluorescence measurements are made on an individual cell basis (Figure 2). Cells that have a fluorescence intensity that is different from control cells are considered to have had some effect to basal autophagy. As is recommended for all methods of monitoring autophagy, complimentary assays should be used to support the findings of the primary assay (4)

Assay Workflow

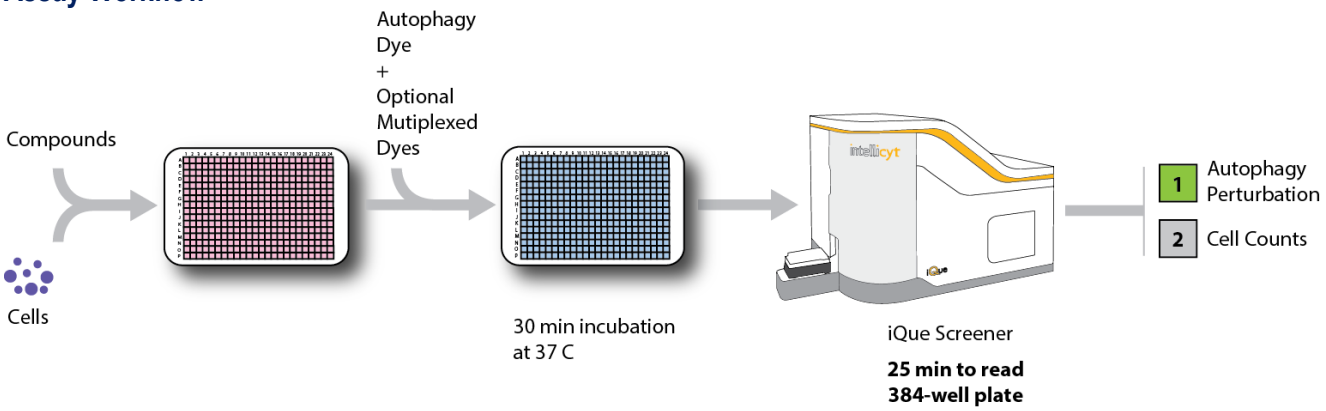

Figure 3. Screening Assay workflow. After cells are treated with compounds in microtiter plates for the desired duration, a cocktail containing the autophagy dye and membrane integrity dye is directly added to the wells of the plate. After a 30 min incubation at 37 °C, the assay plate is ready to read on the iQue Screener. A 384-well plate read can be completed in 25 minutes.

Materials and Methods

Reagents
Autophagy modulating compounds were obtained: 3-Methyladenine, Brefeldin A, Paclitaxel, PI103, Thapsigargin, Wortmannin, Nocodazole, PP242, Tamoxifen, Vinblastine, Rapamycin, Chloroquine, MG132, and Bafilomycin A1.

Cell Culture and Compound Treatment
PC12, U937, A549, H4, HeLa, and HepG2 were all acquired from ATCC and cultured using the ATCC recommended medium and sub-culturing procedures.

A549 experiment.
20 µL of A549 cells at a density of 0.5 million / mL were treated with 100 nM Bafilomycin A1 or 20 µM PP242 in a 384-well plate. After 4 hours incubation (37 °C, 5% CO_2) the cells were stained with autophagy dye and read on the iQue Screener.

Compound Profiling
All cells(0.5M/mL) were treated with compounds and concentrations indicated in Figure 5A for 24 hours. Viability was determined by staining with IntelliCyt's MultiCyt Cell Membrane Integrity reagent. Note that similar determination of cell viability could be conducted with a probe such as 7AAD.

Dose Response Treatments with Chloroquine and PP242
11-point 1:2 serially titrated Chloroquine and PP242 were tested on each of 6 cell lines (0.5M/mL) with top does at 100 µM and 20 µM, respectively. Cells were treated for 24 hours before autophagy staining.

Results and Discussion

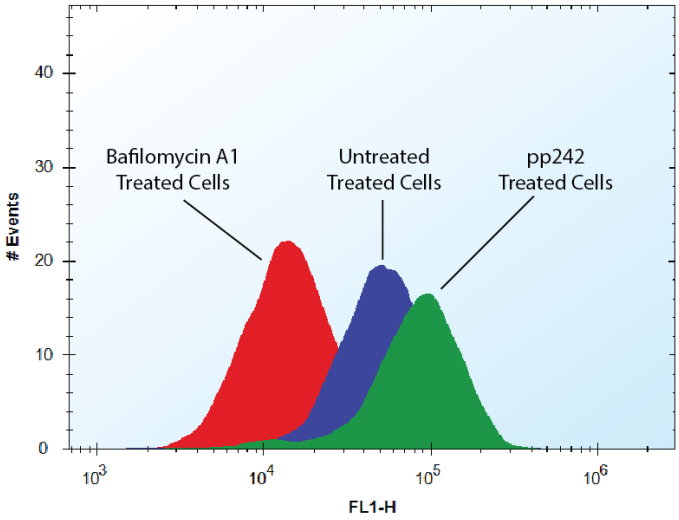

Figure 4. Known modulators of autophagy cause changes in Cyto-ID® Autophagy Dye staining of A549 cells. The frequency distribution of Autophagy Dye staining intensity is shown for A549 cells treated with Bafilomycin A1 (red), PP242 (green), and untreated cells (blue). Treatment with PP242 causes increased staining of cells relative to untreated cells. Treatment with Bafilomycin A1 causes decreased staining of cells relative to untreated cells. See Table 1 for quantitative fluorescence measurements for each treatment type.

Table 1 Error is standard deviation of the mean.

Known modulators of autophagy cause changes in Cyto-ID® Autophagy Dye staining in A549 cells.

	Median Fluorescence Intensity (MFI)	Signal-to-Background
Control	50,311 +/- 3,937	
Bafilomycin A1 (100 nM)	15,240 +/- 2,279	3.3
pp242 (20 µM)	88,204 +/- 7,754	1.8

The Cyto-ID® Autophagy Dye staining response in singleplex was initially evaluated in A549 cells treated with two well-known modulators of autophagy, Bafilomycin A1 and PP242. Bafilomycin A1 is a late-stage autophagy inhibitor, which functions by preventing lysosome acidification and either blocks lysosome fusion with the autophagosome or otherwise prevents acidification of the autolysosome (5). PP242 is known to induce autophagy through mTOR inhibition, a serine/threonine protease that acts as a repressor of autophagy.

A549 cells were incubated for 4 hours in the presence of PP242, Bafilomycin A1, or vehicle-control only (medium + 1% DMSO). Treatment with both autophagy modulators caused changes to autophagy dye staining of cells (Figure 4). Untreated cells had a median fluorescence intensity (MFI) of 50,311, representing the staining of basal-autophagic compartments for this cell line (Table 1). Treatment with Bafilomycin A1 caused a decrease in MFI to 15,240. Treatment with PP242 caused an increase in MFI to 88,204. Using the control-treated cells as the background signal, the signal-to-background ratio for Bafilomycin A and PP242 treatment was 3.3 and 1.8, respectively.

Readers familiar with LC3-II based detection of autophagy modulation will notice that Bafilomycin A1 causes a decrease in signal rather than the expected increase by LC3-II quantification. It is unknown why this discrepancy is observed. It is possible that the autophagy dye does not fluoresce when autophagosomes are not acidified, as should be the case in Bafilomycin A1-treated cells. What is clear from the results is that Bafilomycin A1 treatment causes A549 cells to stain with less Cyto-ID® Autophagy Dye than untreated cells, which scores Bafilomycin A1 as a modulator of autophagy, as expected.

Known modulators of autophagy exhibit different profiles when tested across 6 cell types.

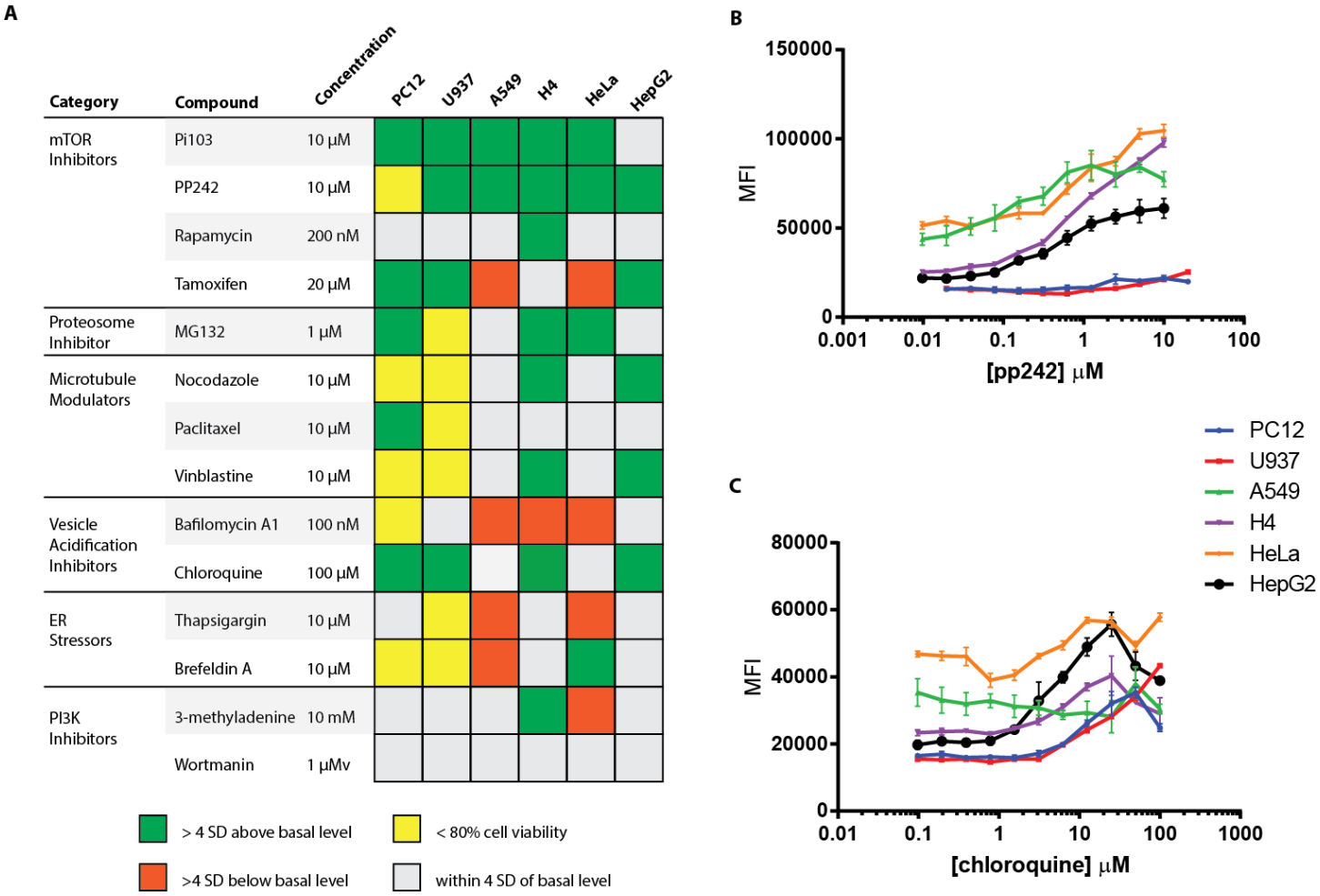

Figure 5. Response profiles for 14 known autophagy-modulators. A) A response was considered significant if it caused Cyto-ID® Autophagy Dye staining to change more than 4 standard deviations from the staining intensity of untreated cells. Responses included both increased staining (green boxes) and decreased staining (red boxes). Cyto-ID® Autophagy Dye responses were not scored when treatments that caused cell viability to drop below 80% (yellow boxes). Dose response treatment with PP242 (B) or Chloroquine (C) in 6 different cell lines.

To further characterize Cyto-ID® Autophagy Dye in different conditions and to assess the compounds effect on autophagy vs.compound toxicity, a set of 14 compounds with known mechanisms of autophagy modulation were profiled for autophagy and membrane integrity responses in treatments of 6 different cell lines: PC12, U937, A549, H4, HeLa, and HepG2. All 14 compounds caused a response that varied by cell line (Figure 5A). A response was considered significant if it differed by greater than 4 standard deviations from the fluorescence of untreated cells. PP242 was closest to a universal autophagy modulator; it caused increased autophagy dye staining in all tested cell lines except for PC12. However, the results from PP242 treatment of PC12 were flagged due to significant

loss of cell viability. Certain compounds caused an increase in Cyto-ID® Autophagy Dye staining in certain cell types, while no-effect or decreases in others. Chloroquine is an example of a compound in this category. It caused increased staining in PC12, U937, H4, and HepG2 cells while causing no change in A549 and HeLa cells.

The profiles for PP242 and Chloroquine were confirmed with dose-response treatments on each cell type. PP242 caused a dose-dependent increase in autophagy staining in all 6 cell types including PC12, which exhibited <80% viability at the top dose (Figure 5B). PP242 responses in PC12 and U937 cells were much weaker than the other cells lines, but still the met the criteria for a significant response at the top doses. The cell lines that scored positive for autophagy modulate with Chloroquine treatment (PC12, U937, H4, and HepG2) were confirmed positive in dose response treatment (Figure 1C).

Conclusions

Taken together, Enzo's Cyto-ID® Autophagy Dye can be multiplexed with the IntelliCyt's MultiCyt Cell Membrane Integrity reagent and measured using the IntelliCyt iQue Screener. This strategy and assay platform offers a convenient, HTS-ready method to screen for modulators of autophagy and cell health. Importantly, the assay does not require an engineered cell line, saving time in assay development and improving relevance of the cellular model used for screening. Mix-and-read chemistry of the dyes requires just a single addition of both dyes to assay volumes, followed by a short incubation. Plates are read, without washing, on the iQue Screener in about 25 minutes for 384 wells. These changes in assay development and screening workflow represent significant improvement in the available strategies for autophagy based drug discovery.

References

1. Levine, B. & Kroemer, G. Autophagy in the Pathogenesis of Disease. *Cell* **132,** 27–42 (2008).
2. Klionsky, D. J. Autophagy as a Regulated Pathway of Cellular Degradation. *Science* **290,** 1717–1721 (2000).
3. Rubinsztein, D. C., Gestwicki, J. E. & Murphy, L. O. Potential therapeutic applications of autophagy : Abstract : Nature Reviews Drug Discovery. *Nature reviews Drug …* (2007).
4. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8,** 445–544 (2012).
5. Klionsky, D. J., Elazar, Z., Seglen, P. O. & Rubinsztein, D. C. Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? *Autophagy* **4,** 849–850 (2008).